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## Short Communication

# Development of catecholamine and cortisol stress responses in zebrafish ☆☆☆



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## ABSTRACT

Both adrenal catecholamines and steroids are known to be involved in the stress response, immune function, blood pressure and energy homeostasis. The response to stress is characterized by the activation of the hypothalamus–pituitary–adrenal (HPA) axis and the sympathetic-adrenomedullary system, though the correlation with activation and development is not well understood. We evaluated the stress response of both cortisol and catecholamines during development in zebrafish. Zebrafish at two different stages of development were stressed in one of two different ways and cortisol and catecholamine were measured. Cortisol was measured by enzyme immune assay and catecholamine was measured by ELISA. Our results show that stress responses are delayed until after the synthesis of both cortisol and catecholamines. These observations suggest that the development of HPA axis may be required for the acquisition of the stress response for cortisol and catecholamines.

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## 1. Introduction

The stress response of cortisol or catecholamines in adult model organisms has been investigated with several stressors: shaking stress, immobilization, and cold or trauma [1–8]. In vivo studies of corticotrophin releasing hormone (CRH) knockout mice, which have reduced corticoid levels, showed interrupted stress-induced increases in both mRNA and protein levels of phenylethanolamine-N-methyltransferase (PNMT), the expression of which is regulated by glucocorticoids and neural input to the adrenal gland [3,4]. Tyrosine hydroxylase (TH) null-mice that lack production of adrenal catecholamines showed reduced levels of plasma corticosterone, and, in the electron micrograph of adrenocortical cells of TH-null mice, internal mitochondrial membranes, where the steroidogenesis takes place, were reduced [9].

The recent studies have described the development of cortisol or catecholamines and the correlation with the various stressors [10–15]. In zebrafish, the ability to synthesize cortisol by the interrenal organ, the adrenal equivalent, starts after hatch, though the response with cortisol elevation induced by stress does not exist at that time [10–12]. This discrepancy may be caused by the immaturity of the HPA-axis and its ability to respond to stress, as up-regulation of the expression of melanocortin type 2 receptor (MC2R) transcripts, which precedes cortisol synthesis, is already seen at the time around hatch [10–12]. The catecholamines are detectable from 1 h post fertilization (hpf) and significantly increased from 2 days post fertilization (dpf) toward 5 dpf in zebrafish development [14].

In this study, to better understand the stress response of the catecholamines and the correlation between cortisol and catecholamines during development, we analyzed both catecholamine and cortisol levels at several developmental stages under control conditions and after acute stressors using the zebrafish, *Danio rerio*, a powerful model for understanding organ development due to its ease of genetic and molecular manipulation, transparent embryos, and large number of progeny that provide statistical power [16,17].

## 2. Materials and methods

### 2.1. Zebrafish breeding and embryo maintenance

Wild-type zebrafish (AB line or Hybrid line) were maintained at the UCLA Zebrafish Core Facility. Embryos were obtained by natural spawning and cultured in culture aqua medium (fishwater) at 28 °C using standard zebrafish husbandry techniques [18]. Zebrafish were maintained in accordance with the Guide for the Care and Use of Laboratory Animals [19], and the studies were approved by the UCLA institutional committee on animal care.

### 2.2. Cortisol extraction and measurement

Wild type zebrafish embryos were cultured in fishwater with standard procedures [17]. A hundred embryos or larvae were gently collected into a 15 ml tube at 48 or 96 hpf, respectively. Stressed samples were prepared in one of the two ways: 30 s of hand swirling in 5 ml fishwater or 30 revolutions of roller swirling using a tube shaker/rotator (LABQUAKE, model 4002110; Barnstead International, IO, USA) at 8 rpm in 10 ml fishwater. After being stressed, samples were placed at 28 °C for 5 min and then on ice immediately, and embryos were transferred to a 1.5 ml tube and stored at –80 °C until use. For extraction, we followed the procedure as previously reported [13]. Cortisol enzyme immunoassay (EIA) (Cortisol EIA kit, Cat No. 500360; Cayman Chemical, MI, USA) was performed to determine the cortisol levels for each sample in duplicate.

### 2.3. Catecholamine extraction, acylation and measurement

Wild type zebrafish embryos were cultured in fishwater with standard procedures [17]. A hundred and fifty embryos at 48 hpf or a hundred larvae at 98 and 120 hpf were gently collected into a 15 ml tube and placed in the 28 °C incubator for 15 min. Stressed samples were prepared as follows: 30 s of hand swirling in 5 ml fishwater or 15 revolutions of roller swirling using the shaker/rotator in 10 ml fishwater. Tricaine was added soon after the stress to make a 0.02% final concentration in fishwater

with both control and stressed samples. Samples in tricaine were moved to 1.5 ml tubes, placed on dry ice immediately and then stored at  $-80^{\circ}\text{C}$  until used. For extraction, two hundred microliters of 10 mM Tris-HCl (pH 7.4) were added to each sample and then samples were homogenized for 30 s using a rotor-stator homogenizer. Homogenized samples were centrifuged at  $10,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . Whole supernatant from each sample was used for extraction and acylation according to manufacturer's instructions. Finally, the catecholamine measurements were taken for each sample in duplicate using a 2-Cat ELISA kit (2-Cat ELISA Fast Track, BA E-6500; Rocky Mountain Diagnostics, Inc., CO, USA).

### 3. Results and discussion

The recent studies demonstrated that the synthesis of cortisol begins about 48 hpf, around the time of hatch, and stress-induced cortisol elevation occurs from 97 hpf after the development of the HPA-axis in zebrafish [10–12]. Our data confirmed obvious stress induced cortisol elevation at 97 hpf with both roller and hand swirling (Fig. 1A, Student's *t*-test, *p* value =  $1.06\text{E-}06$ , Control vs. Roller swirling; Student's *t*-test, *p* value =  $8.5\text{E-}07$ , Control vs. Hand swirling). Moreover, mild, but statistically significant stress induced cortisol elevations were observed at 48 hpf with roller swirling (Fig. 1A, Student's *t*-test, *p* value = 0.009). These results suggest that the mildly increased cortisol, which is induced by stress at 48 hpf, may be due to the direct response of the adrenal gland, since the HPA axis, which mediates the stress-activated cortisol response, is not developed at this stage [10–12].

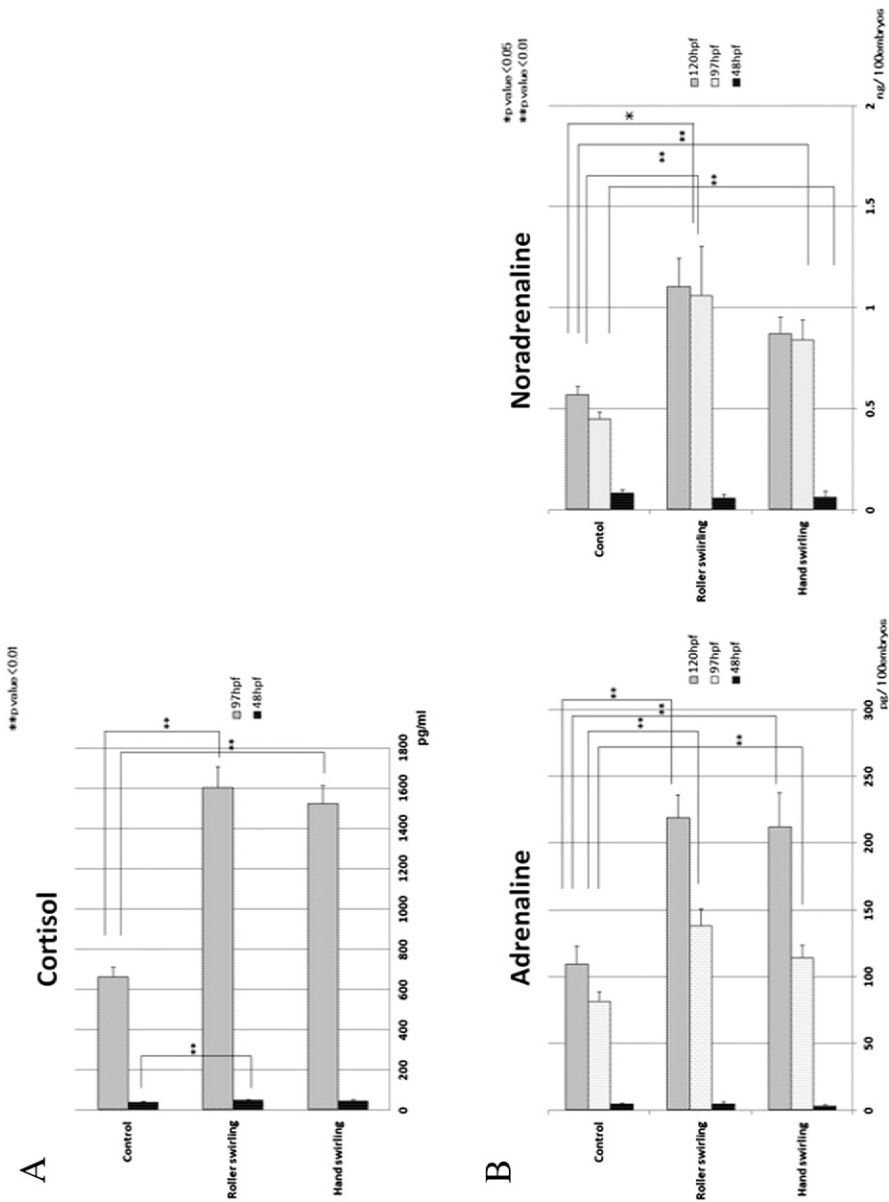
As adrenal catecholamine release is also regulated by stress and other factors, we evaluated natural and stress induced elevations of catecholamines. In situ hybridization for dopamine beta hydroxylase (dbh), which hydroxylates dopamine to noradrenaline, is observed in locus coeruleus (LC) and arch associated nerve (AAN) at 24 hpf and strongly expressed in the intrarenal region at 48 hpf, and at the hatch stage (data not shown). Steele et al. showed both adrenaline and noradrenaline were detectable from 1 hpf and levels of catecholamines were gradually increased from 1 hpf to 5 dpf [14]. Our data show that adrenaline and noradrenaline levels were increased approximately 24 fold and 7 fold from 48 hpf to 120 hpf respectively (mean levels of adrenaline: 4.61 pg and 109.23 pg/100 embryos at 48 and 120 hpf, respectively; mean levels of noradrenaline: 0.082 ng and 0.567 ng/100 embryos at 48 and 120 hpf, respectively) (Fig. 1B).

Hand and roller swirling were used as stressors at 48, 97 and 120 hpf. Increased levels of catecholamines were not observed after stress with either hand or roller swirling at 48 hpf (Fig. 1B). Stress responses of adrenaline and noradrenaline were detected at 97 hpf (Fig. 1B) and the strengths of the stress responses were increased for adrenaline between 97 and 120 hpf (ratio of adrenaline in hand swirling/control and roller swirling/control: 1.41, 1.70 at 97 hpf and 1.94, 2.00 at 120 hpf, respectively) (Fig. 1B). The type of the stress did not show a statistically significant difference in levels of catecholamines by Student's *t*-test (Fig. 1B).

In conclusion, our studies revealed that although both cortisol and catecholamines were generated around hatch, significant responses to stress were delayed during the development of zebrafish. Activation of the HPA axis with release of CRH from the paraventricular nucleus (PVN) of the hypothalamus, followed by ACTH and glucocorticoid release is important in the homeostasis of the stress response. Noradrenaline is thought to be a potent stimulator of CRH neurons in the PVN [7]. We suspect not only the sympathetic-adrenomedullary system, but also the HPA axis may be required for the development of the stress response for catecholamines. Further studies are needed to describe the precise correlation between HPA axis and catecholamines during the development of the stress responses.

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**Fig. 1.** Cortisol and catecholamines levels among various stressed conditions during early development in zebrafish (\*\* =  $p < 0.01$ ). (A) Cortisol levels in zebrafish embryos with acute stress were determined at 48 or 97 hpf. The baseline cortisol level was low at 48 hpf and significantly increased by 97 hpf. Stress-activated elevation of cortisol was observed at 97 hpf ( $p = 1.1E-06$ ; Control vs. Roller swirling,  $p = 8.5E-07$ ; Control vs. Hand swirling), as previously reported [10–12]. In stressed samples with roller swirling, the cortisol level was slightly elevated compared to control at 48 hpf ( $p = 0.009$ ; Control vs. Roller swirling). Values are means  $\pm$  SEM.  $n = 15$  (control),  $n = 16$  (roller swirling),  $n = 17$  (hand swirling). (B) Adrenaline and noradrenaline levels in zebrafish embryos with acute stress were determined at 48, 97 or 120 hpf. The baseline catecholamine level were low at 48 hpf and gradually increased toward 120 hpf. Stressed-activated elevation of catecholamines were observed at 97 and 120 hpf (97 hpf: Adrenaline,  $p = 0.008$ ; Control vs. Roller swirling,  $p = 0.0006$ ; Control vs. Hand swirling, Noradrenaline,  $p = 0.003$ ; Control vs. Roller swirling,  $p = 0.002$ ; Control vs. Hand swirling, 120 hpf: Adrenaline,  $p = 1.26E-05$ ; Control vs. Roller swirling,  $p = 0.0005$ ; Control vs. Hand swirling, Noradrenaline,  $p = 0.002$ ; Control vs. Roller swirling,  $p = 0.003$ ; Control vs. Hand swirling). There are no statistically significant differences between control and stressed samples at 48 hpf (Adrenaline,  $p = 0.99$ ; Control vs. Roller swirling,  $p = 0.20$ ; Control vs. Hand swirling, Noradrenaline,  $p = 0.49$ ; Control vs. Roller swirling,  $p = 0.20$ ; Control vs. Hand swirling). Values are means  $\pm$  SEM. Adrenaline;  $n = 41$  (control),  $n = 31$  (roller swirling),  $n = 38$  (hand swirling), Noradrenaline;  $n = 40$  (control),  $n = 33$  (roller swirling),  $n = 35$  (hand swirling).